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A chimeric virus created by DNA shuffling of the capsid genes of different subtypes of porcine circovirus type 2 (PCV2) in the backbone of the non-pathogenic PCV1 induces protective immunity against the predominant PCV2b and the emerging PCV2d in pigs

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Running title: Shuffled PCV2 protects against emerging strains

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Abstract

Porcine circovirus type 2 (PCV2) is the primary causative agent of porcine circovirus-associated disease (PCVAD). Available commercial vaccines all target~~ the~~ PCV2a subtype, although the circulating predominant subtype worldwide is PCV2b, and the emerging PCV2d subtype is also increasingly associated with PCVAD. Here we molecularly bred genetically-divergent strains representing PCV2a, PCV2b, PCV2c, PCV2d, and “divergent PCV2a~~PCV2e~~” subtypes by DNA-shuffling of the capsid genes to produce a chimeric virus representing PCV2 global genetic diversity. When placed in the PCV2a backbone, one chimeric virus (PCV2-3cl14) induced higher neutralizing antibody titers against different PCV2 subtypes. Subsequently, a candidate vaccine (PCV1-3cl14) was produced by cloning the shuffled 3cl14 capsid into the backbone of the non-pathogenic PCV1. A vaccine efficacy study revealed that chimeric virus PCV1-3cl14 induces protective immunity against challenge with PCV2b or PCV2d in pigs. The chimeric PCV1-3cl14 virus is a strong candidate for a novel vaccine in pigs infected with variable PCV2 strains.

Keywords: Porcine circovirus type 2 (PCV2); porcine circovirus-associated disease (PCVAD); DNA shuffling; capsid; vaccine

Introduction

Porcine circovirus (PCV) is a small, non-enveloped, single-stranded DNA virus which belongs to the family *Ciroviridae* (1). PCV type 1 (PCV1) was originally identified as a cell culture contaminant of the porcine kidney cell line PK-15 in the 1970's, and was later found to be non-pathogenic in pigs (2, 3). In 1997, a pathogenic variant designated as PCV type 2 (PCV2) was identified in wasting piglets shortly after weaning (2, 4-9). As more cases were identified worldwide, PCV2 was determined to be the primary causative agent of porcine circovirus-associated disease (PCVAD), which includes a broad spectrum of clinical symptoms such as wasting, reproductive failure, respiratory signs and enteritis, and PCV2 may also have a role in the porcine dermatitis and nephropathy syndrome (10).

PCV2 is one of the most economically devastating viral pathogens to affect the global pig industry to date, and vaccination has been an effective strategy to reduce the economic losses associated with PCV2 infection (11). Currently, all commercially available inactivated or subunit vaccines ~~target the~~consist of a single PCV2a ~~subtype~~ capsid antigen (11-14). However, since 2005, a new subtype, PCV2b, has taken over as the most prevalent PCV2 strain associated with PCVAD cases in the U.S. and other countries (15-17). In addition, newly emerging PCV2d strains (previously referred to as "mutant PCV2b"), have been identified in an increasing number of cases in vaccinated herds worldwide, leading to the speculation by some that the emerging PCV2d strains are able to overcome vaccine protection (18-20). A recent study showed that animals vaccinated with recombinant PCV2a capsid protein had lower viral loads and generated higher neutralizing antibodies against a PCV2d-1 strain than vaccination with either a

PCV2b or homologous PCV2d-1 recombinant capsid protein, suggesting that PCV2 capsid immunogenicity varies (21). However this could not fully explain how PCV2d infections are emerging in PCV2a vaccinated herds.

Until recently, only three PCV2 subtypes were recognized, including PCV2a, PCV2b, and PCV2c, the last of which was identified in Denmark during the 2000's, is recognized but not very prevalent (22, 23). While the majority of the PCVAD cases in the United States are now associated with PCV2b, the emerging PCV2d subtype has been slowly increasing in the U.S since its initial discovery in 2012 and is now more prevalent than PCV2a (24). Although the exact reason for the emergence of PCV2d remains unclear, it can be commonly found in vaccinated herds, leading to the speculation of either reduced protection against this emerging PCV2d or vaccination failure of individual animals (18). While the introduction of PCV2a based vaccine strategies has resulted in a drastic decline in PCV2 prevalence (25), the increased genetic diversity of PCV2 strains is concerning, and is suggestive of selective pressure promoting genetic diversity. In fact, a recent report has demonstrated the increasing genetic diversity amongst the PCV2d subtype, as the majority of isolates identified from 1999-2011 can be classified under the subclade "PCV2d-1," and the majority of isolates identified recently, from 2006-2014, diverge from the PCV2d-1 subclade and are now designated "PCV2d-2" (24). In addition, *in vitro* evidence suggests distinct antigenic differences among PCV2 subtypes, which may help explain the emergence of new strains (26, 27). Therefore, in order to address the concern of emerging PCV2d as well as the predominant PCV2b now circulating in global swine herds, as well as the possibility for the generation of increasingly divergent PCV2 strains that cannot be controlled by vaccination with a

PCV2a antigen alone, future vaccine strategies should focus on broadening the protection of a single vaccine by targeting emerging strains such as PCV2d and the predominant PCV2b subtype.

DNA shuffling has been shown to be a powerful tool to introduce genetic diversity into the virus of interest (28, 29). In fact, recently our group has successfully shuffled the structural genes of porcine reproductive and respiratory syndrome virus (PRRSV) and developed chimeric virus vaccine candidates with broadly protective properties against heterologous PRRSV strains (30-33). Therefore, in the present study we aimed to molecularly breed by DNA shuffling the capsid genes of 5 genetically diverse PCV2 subtypes including PCV2a, PCV2b, PCV2c, PCV2d and a capsid sequence representing a recently identified divergent PCV2a virus previously referred to as “PCV2e.” ~~“PCV2e”~~ The “PCV2e” genotype was ~~(~~originally identified by phylogenetic analysis of the capsid sequence (34), but was later determined be included in the~~or divergent~~ PCV2a genotype based on full sequence phylogenetic analysis (35)~~)~~. While the “PCV2e” strains identified are not divergent enough from PCV2a strains to be referred to as their own genotype, this strain was included in this study to increase genetic diversity of the PCV2 capsids utilized for DNA shuffling, and will be referred to as “divergent PCV2a” in this paper to separate it from the classic PCV2a strain used in this study. In ~~in~~ order to create a chimeric virus that can induce broad cross-protection against different PCV2 subtypes especially the emerging PCV2d and the currently predominant circulating PCV2b.

We were able to successfully generate four viable chimeric viruses with shuffled capsid gene sequences in the backbone of PCV2a. An *in vivo* pilot study was first conducted in pigs to assess the infectivity and cross-neutralizing activities of these 4 chimeric viruses. The chimeric virus (3cl14) exhibiting the highest level of cross-neutralizing activity against different PCV2 subtypes were subsequently selected for a challenge and efficacy study in pigs against the currently predominant circulating PCV2b strain as well as the emerging PCV2d strain. We demonstrated that the capsid-shuffled chimeric virus 3cl14 induces protective immunity in conventional pigs against challenges with both PCV2b and PCV2d.

Materials and Methods

Cells*:* A subclone of the PK-15 cell line that is free of PCV1 contamination was produced previously by end-point dilution of PK-15 cells (ATCC CCL-33) (36). This subclone PK-15 cell line was cultured in Minimal Essential Medium (MEM) supplemented with 10% Fetal Bovine Serum (FBS) and antibiotics and was used in the serum virus neutralization assay and to propagate all virus stocks for this study.

DNA shuffling of the capsid genes from 5 different PCV2 subtypes*:* The capsid gene sequences representing each of the 5 genetically-diversified PCV2 subtypes were selected for DNA shuffling, including PCV2a (strain 40895, GenBank accession number AF264042), PCV2b (strain NC16845, accession number GU799576), PCV2c (accession number EU148503), PCV2d-1 (accession number AY181947), and "~~PCV2e~~divergent PCV2a" (accession number EF524533). The PCV2a and PCV2b strains were isolated from U.S. pigs and described previously (12, 37), while the PCV2c, PCV2d-1, and “divergent PCV2a~~PCV2e~~” capsid genes were synthesized by GenScript (PIscataway, NJ).

Traditional DNA shuffling was used to shuffle the 5 different PCV2 capsid genes essentially as previously described for PRRSV (31), with slight modifications. Briefly, the capsid gene DNAs from each of the five PCV2 strains were mixed in equimolar amounts with a total of 5 µg DNA and diluted in 50 µl of 50 mM Tris-HCl (pH 7.4) and 10 mM MgCl2. The mixture was incubated at 15°C for 3 min with 0.15 U of DNase I (Sigma). DNA fragments ranging from 50 to 150 bp in size were purified from 2% agarose gels, and subsequently added to the Pfu PCR mixture consisting of 1X Pfu buffer, 0.2 mM each deoxynucleoside triphosphate (dNTP), and 0.06 U Pfu polymerase. A PCR program without using primers (95°C for 4 min; 40 cycles of 95°C for 30s, 60°C for 30s, 57°C for 30s, 54°C for 30s, 51°C for 30s, 48°C for 30s, 45°C for 30s, 42°C for 30s, and 72°C for 2 min; and finally, 72°C for 7 min) was performed to reassemble the digested DNA fragments. Subsequently, specific primers flanking the shuffled PCV2 capsid region, UniRep-F and 2aORF2-R (**Table S1**), were used to amplify the shuffled PCV2 capsid using Pfu Ultra II Hotstart PCR Master Mix (Agilent Technologies) per the manufacturer’s instructions (95°C for 4 min, 10 cycles of 95°C for 30s, 50°C for 30s, 72°C for 30s, 25 cycles of 95°C for 30s, 54°C for 30s, 72°C for 30s, and finally 72°C for 7 min).

***Construction of infectious DNA clones of chimeric PCV2a and PCV1 viruses with shuffled PCV2 capsid genes**:* The shuffled capsid gene product libraries were cloned into the blunt end cloning vector, pCR-Blunt II, using the Zero Blunt® TOPO® PCR

Cloning kit (Life Technologies, Carlsbad), per manufacturer's instructions. Selected clones were sequenced and analyzed for DNA shuffling efficiency, and well-shuffled capsid genes containing regions from all 5 PCV2 subtypes were amplified and subsequently cloned into the infectious DNA clone backbone of the PCV2a strain 40895 by fusion PCR, essentially as previously described (38). Briefly, the shuffled PCV2 capsids were amplified using primers UniRep-F and 2aORF2-R (**Table S1**). The PCV2a infectious DNA clone backbone sequence was amplified in two fragments that flank the PCV2 capsid region using primers SacII-uni-F and UniRep-R, and primers 2aORF2F and SacII-uni-R, for PCV2a fragments 1 and 2, respectively (**Table S1**). All three PCR reactions were performed using ACCUZYME MIX™ (Bioline) at 95°C 10 min, 35 cycles of 95°C for 30s, 54°C for 30s, and 68°C for 1.5 min. The first fusion PCR was performed with the PCV2 fragment 1 and the shuffled PCV2 capsid sequence using the external primers SacII-uni-F and 2aORF2-R. Subsequently, a second fusion PCR reaction was performed with the product of the first fusion PCR reaction and the PCV2a fragment 2, using the external primers SacII-uni-F and SacII-uni-R (**Table S1**). All fusion PCR reactions were performed using ACCUZYME MIX™ at 95°C 10 min, 35 cycles of 95°C for 30s, 60°C for 30s, and 68°C for 4 min. The full-length chimeric PCV2a containing each individual shuffled PCV2 capsid was amplified, and cloned into the pCR-Blunt II TOPO plasmid using the Zero Blunt® cloning kit to produce infectious DNA clones of chimeric PCV2a with shuffled capsid genes.

The shuffled PCV2 capsid 3cl14 was cloned into the infectious DNA clone backbone of the non-pathogenic PCV1 to create the vaccine candidate PCV1-3cl14 by a similar fusion PCR protocol. Briefly, the shuffled PCV2 capsid 3cl14 was amplified using primers PCV1-BB-F and PCV1-DS-ORF2-R (**Table S1**). The infectious DNA clone PCV1 backbone sequence was amplified from the PBSK+ plasmid containing PCV1 in two fragments that flank the PCV1 capsid region using primers M13F (-20) and PCV-BB-R, and primers PCV-DS-ORF2-F and M13R, for PCV1 fragments 1 and 2, respectively (**Table S1**). All three PCR reactions were performed using Platinum® PCR Supermix (Thermo Scientific) at 94°C 3 min, 35 cycles of 94°C for 30s, 55°C for 30s, and 68°C for 1 min. Fusion PCR was performed first with the PCV1 fragment 1 and the shuffled PCV2 capsid 3cl14 fragment using the external primers M13F and PCV1-DS-ORF2-R (**Table S1**). A second fusion PCR reaction was performed with the product of the first fusion PCR reaction and PCV1 fragment 2, using the external primers M13F and M13R. The full-length chimeric PCV1 virus containing the shuffled capsid 3cl14 was cloned into pCR-Blunt II TOPO using the Zero Blunt® cloning kit to produce the infectious DNA clone of vaccine candidate chimeric PCV1 virus 3cl14.

***Preparation of virus stocks**:* The infectious virus stocks of PCV2b strain NC16845, U.S. PCV2d-2 strain JX535296, and each of the PCV2a capsid-shuffled chimeric viruses were produced by transfecting PK-15 cells with concatemerized viral genomes from the respective infectious DNA clones. Briefly, the respective PCV2 genomes were excised from pCR-Blunt II TOPO by SacII digestion, concatemerized, and transfected into PK-15 cells to determine the viability and infectivity by immunofluorescence assay (IFA) as previously described (36, 37, 39). The virus stocks for the chimeric PCV1-2a and chimeric PCV1 containing shuffled 3cl14 capsid (PCV1-3cl14) were prepared similarly as described above except that the viral genome was excised from the pCR-Blunt II TOPO vector by digestion with KpnI prior to concatemerization.

Determination of the infectivity and cross-neutralizing activities of the PCV2 capsid-shuffled viruses*:* To initially identify viable PCV2 capsid-shuffled viruses with improved cross-neutralizing activities against different PCV2 subtypes, we first conducted a pilot pig infection study with a limited number of animals (n=3). A total of 18, 4-week-old, cross-breed conventional pigs were purchased from a commercial farm that is known to be free of PRRSV and M. hyo without active PCV2 circulation as determined by regular PCV2 PCR on selected batches of pigs. Sows have low amounts of antibodies against PCV2 or are seronegative and we selected litters from negative sows without cross-fostering. The piglets were randomly assigned to six groups of 3 pigs each, and each group of pigs was housed separately. Prior to inoculation, each pig was weighed, bled, and confirmed to be negative for PCV2 by PCR and serology. Five groups were inoculated intramuscularly each with 5 ml ($10^{3.66}$ $TCID_{50}$/mL) of either chimeric virus PCV1-2a or one of the four PCV2 capsid-shuffled viruses (PCV2-3cl13, PCV2-3cl14, PCV2-3cl4-2, or PCV2-3cl12-2). One group was mock-inoculated similarly with 5 mL of PBS buffer (**Table 1**). Blood was collected weekly, and animals were monitored for seroconversion to PCV2 capsid antibodies by ELISA and evidence of PCV2 infection by qPCR. Animals were necropsied at 56 days post-infection (dpi). The weekly serum samples were used to perform serum virus neutralization test against strains representing different PCV2 subtypes (data not shown for 0-49 dpi). The animal study was approved by Virginia Tech IACUC.

Serum virus neutralization assay: Serum samples collected from infected pigs were tested for neutralizing antibody titers against the wild-type PCV2a, PCV2b, PCV2d-1, and PCV2d-2 strains by IFA. Briefly, the serum samples were serially diluted 1:2 in PBS and mixed with 150 $TCID_{50}$ of PCV2a, PCV2b, PCV2d-1, or PCV2d-2 virus stocks, respectively, at an equal volume ratio and incubated for 1 hr at 37˚C. The serum-virus mixture was then added to PK-15 cells in a 96 well plate in duplicate. After 72 hrs incubation at 37˚C, an IFA was preformed using pig sera against PCV2a diluted 1:1000, as the primary antibody and FITC-conjugated goat anti-pig IgG (KPL) diluted 1:50 as the secondary antibody. The 50% serum neutralizing antibody titers were determined as the highest dilution at which there was 50% or greater reduction in virus titer compared with the average of the serum from PBS control pig group at that dilution.

Vaccination efficacy and challenge study in conventional pigs: The virus containing shuffled capsid 3cl14 in the backbone of PCV2a induced significantly higher neutralizing antibody responses against different PCV2 strains. Therefore, the shuffled capsid sequence 3cl14 was subsequently cloned into the infectious DNA clone backbone of non-pathogenic PCV1 to produce a PCV1-3cl14 shuffled capsid chimeric virus as the vaccine candidate. Subsequently, a pig challenge study was conducted to evaluate the efficacy of the candidate PCV1-3cl14 chimeric virus vaccine against infection with currently predominant circulating PCV2b as well as the emerging PCV2d-2. This experiment was a subset of a larger study. However, wild type exposure prevented completion and analysis of other groups.

Briefly, a total of 32, 3-week-old, cross-breed conventional pigs were purchased from a commercial farm that is known to be free of PRRSV and M. *hyopneumoniae*, and is negative for PCV2. The animal study was approved by Iowa State University IACUC as well as by Virginia Tech IACUC. The piglets were randomly assigned to 4 groups of 8 pigs each. Prior to inoculation, each pig was weighed, bled, and confirmed to be negative for PCV2. Groups 1 and 2 pigs were each vaccinated intramuscularly (IM) in the neck region with 5 ml of the candidate PCV1-3cl14 chimeric virus vaccine ($10^{3.7}$ $TCID_{50}$/mL per pig). Groups 3 and 4 pigs were each mock-vaccinated IM with 5 ml PBS buffer (**Table 2**). All animals were monitored daily for clinical signs including wasting, respiratory distress, and behavioral changes such as lethargy and inappetence. Blood samples were collected prior to inoculation, and weekly thereafter from each pig through 42 days post-vaccination (dpv).

At 42 dpv, groups 1 (vaccinated) and 3 (mock-vaccinated) pigs were each challenged with $10^{4.8}$ $TCID_{50}$ (2.5 ml intranasally and 2.5ml IM) of the PCV2b NC16845 virus strain, and groups 2 (vaccinated) and 4 (unvaccinated) were each similarly challenged with $10^{4.8}$ $TCID_{50}$ of the PCV2d-2 JX535296 virus strain. Blood samples were collected weekly through 20 days post-challenge (dpc) (or 62 dpv), at which time all pigs were weighed and necropsied. A panel of serum and tissue samples was collected for quantification of viral DNA loads and for histological examination of PCV2-associated lesions.

***Gross pathology and histopathology evaluation**:* Necropsies were performed at 20 dpc on all pigs in a treatment status blinded fashion. Estimates of macroscopic lung lesions (ranging from 0 to 100% of the lung affected) and lymph node size (ranging from 0 [normal] to 3 [four times the normal size]) were obtained for each pig (40, 41). Sections of lung, lymph nodes (superficial inguinal, mediastinal, tracheobronchial, and mesenteric), tonsil, heart, thymus, kidney, spleen, and liver were collected during necropsy and processed routinely for histological examination and PCV2 immunohistochemistry (IHC) (Iowa State University Veterinary Diagnostic Lab). Also, samples of tracheobronchial lymph node (TBLN) were collected from each pig for DNA extraction and quantification of PCV2 viral genomes by real-time quantitative PCR. Microscopic lesions in the lymphoid tissues, lungs, heart, liver, kidney, ileum, and colon were scored in a treatment status blinded manner, as described previously (40). Specifically, lymph nodes, spleen, and tonsil were evaluated for presence and degree of lymphoid depletion and histiocytic replacement.

Quantitative PCR to quantify viral DNA loads in serum and tissues

For both animal experiments we used a previously published protocol to extract DNA from serum and lymph node samples and a previously published qPCR SYBR green assay to quantify viral loads in these samples (37, 42). For the pilot infection study (**Table 1**) and for the challenge experiment (**Table 2**), PCV2 specific primers were used to amplify a conserved region spanning the origin of replication and a portion of the replicase gene, as previously reported ***(37),*** using primers PCV2-83F and PCV2-83R (**Table S1**). For the detection of the PCV1-3cl.14 vaccine strain in the challenge study (**Table 2**), primers PCV1-qRepF and PCV1-qRepR primers (**Table S1**) were used to amplify only the PCV1 backbone based vaccine virus DNA.

Serology: A PCV2-specific ELISA using PCV2a capsid antigen (Iowa State University Veterinary Diagnostic Lab) was used to detect anti-PCV2 ORF2 IgG in each serum sample as previously described (43).

Sequence confirmation of virus recovered from infected pigs: DNA extracts from serum samples collected at 20 dpc from selected pigs in each group were tested by PCR for PCV2 capsid sequences, and the amplified PCR products were sequenced to verify that the virus recovered from the infected pigs was the same virus inoculated into the animals. PCR primers Unirep-F and 2aORF-2 were used to amplify the PCV2 capsid gene in these samples using the same PCR program as described above for cloning (**Table S1**). Additionally, DNA extracts of TBLN tissues from selected pigs in each group were also tested to confirm that the virus detected by PCR from infected pigs was the same virus that was inoculated into the animals. PCV2b was amplified and sequenced using primers specific for PCV2b as previously described (37). The PCV2d-2 vDNA was amplified and sequenced using the same forward primer as for PCV2b and a PCV2d-specific reverse primer NB-56-m2b (**Table S1**).

Statistical Analysis: Statistical analysis was performed using Prism v6.0 (Graphpad, La Jolla CA). A one-tailed t-test was used to analyze statistical significance between two groups, while a one-way ANOVA and then t-tests corrected for multiple comparisons were used to determine significance between three or more groups.

Results

Generation of infectious chimeric viruses containing the shuffled capsid from 5 genetically distinct PCV2 strains: Traditional DNA shuffling was used to molecularly breed the capsid genes from five genetically distinct PCV2 strains representing different subtypes PCV2a PCV2b, PCV2c, and PCV2d-1, as well as “divergent PCV2a~~PCV2e~~” ~~(divergent PCV2a)~~ (**Fig. 1**). Although the general consensus is that previously classified “divergent PCV2a~~PCV2e~~” virus isolates do not diverge enough from identified PCV2a strains to be considered their own subtype (35), a “divergent PCV2a~~PCV2e~~” capsid sequence was chosen to help increase the genetic diversity of the resulting shuffled capsid. The capsid gene sequences from these 5 strains were shuffled using DNase I digestion and reassembled by PCR without primers. A PCR product of the expected size was then generated after a second round of PCR with specific primers spanning the capsid gene. The shuffled capsid gene library was then cloned into the infectious clone backbone of PCV2a (strain 40985) to screen for viable viruses. Of the more than 50 clones with “well-shuffled” capsids (containing regions from all 5 parental PCV2 strains), only 4 of them successfully rescued infectious virus when transfected into PK-15 cells (data not shown).

The four viruses with shuffled capsids contain a range of combinations of the genetic signatures of PCV2 genomes from all 5 parental strains (**Fig. 1**). The majority of the unique amino acid signatures introduced into the shuffled capsids originated from PCV2c, which is not surprising since PCV2c is the most genetically distinct of the 5 parental strains, based on a phylogenetic analysis (**Fig. 2**). Therefore, we demonstrated here that traditional DNA shuffling successfully generated viable infectious chimeric viruses with shuffled capsid genes from 5 different PCV2 subtypes.

PCV2-3cl14 with shuffled capsid genes induces cross-neutralizing antibodies against different PCV2 subtypes*:* To determine the viability and screen for the best virus with shuffled capsids for subsequent challenge and efficacy study, we experimentally infected conventional pigs with each of the four viruses (PCV2-3cl13, PCV2-3cl14, PCV2-3cl4_2, and PCV2-3cl12) as well as with the chimeric PCV1-2a virus (12). Serum samples were collected prior to infection and weekly thereafter, and all animals were monitored for serconversion to PCV2a capsid by an ELISA (**Table 1**). All animals experimentally inoculated with PCV1-2a or with PCV2-3cl14 seroconverted to PCV2 antibodies by 49 days post-inoculation (dpi), however only 2 out of 3 animals in the PCV2-3cl12_2 and 1 of 3 pigs inoculated with either virus PCV2-3cl4 or PCV2-3cl4_2 were seropositive at 49 dpi (**Table 1**).

Serum samples collected from 56 dpi were tested by a serum virus neutralization assay in PK15 cells for cross-neutralizing antibodies against wild-type PCV2a, PCV2b, a PCV2d-1, and PCV2d-2 virus strains (**Fig. 3**). The neutralization assay was not performed against the parental PCV2c and divergent PCV2a~~PCV2e~~ strains because PCV2c viruses have not associated with PCV2-induced disease and attempts to grow the divergent PCV2a~~PCV2e~~ wild type virus in PK-15 cells was unsuccessful in our hands (data not shown). Infections of pigs with 3 PCV2 viruses with shuffled capsid genes (PCV2-3cl13, PCV2-3cl4_2, and PCV2-3cl12) did not induce higher levels of neutralizing antibody when compared to the chimeric PCV1-2a virus which is the basis for the current Fostera™ PCV commercial vaccine. However, infection of pigs with the chimeric virus PCV2-3cl14 with shuffled capsid genes from different PCV2 subtypes induced significantly higher neutralizing antibody titers against PCV2a and PCV2d-2 when compared to PCV1-2a ($p<0.05$) (**Fig. 3**). In addition, although not statistically significant, the chimeric virus PCV2-3cl14 also induced higher levels of neutralizing antibody than the PCV1-2a against both PCV2b and PCV2d-2. Taken together, this pilot animal study suggests that the viruses with shuffled capsid genes are viable and infectious in pigs, and that one shuffled capsid virus PCV2-3cl14 induces significantly higher levels of neutralizing antibodies against genetically distinct PCV2 strains when compared to the other chimeric viruses as well as to the PCV1-2a vaccine virus. Therefore, the virus PCV2-3cl14 was selected for the subsequent challenge and efficacy study in pigs to evaluate its potential use as a novel vaccine.

The chimeric virus PCV1-3cl14 induces protective immunity in conventional pigs against challenge with PCV2b and PCV2d-2. PCV2a is the genomic backbone for the virus PCV2-3cl14. Therefore, in order to produce a novel vaccine candidate, we subsequently transferred the shuffled capsid gene from the virus PCV2-3cl14, identified in the initial cross-neutralization study, to the genomic backbone of the non-pathogenic PCV1 to produce a new chimeric virus PCV1-3cl14 with a shuffled capsid. To assess whether the chimeric virus PCV1-3cl14 vaccine candidate protects against challenge with different PCV2 subtypes, two groups of pigs (n=8) were each vaccinated with the PCV1-3cl14 chimeric virus, and another two groups of pigs (n=8) were mock-vaccinated with PBS as controls (**Table 2**). Blood samples were taken weekly and animals were monitored for seroconversion to PCV2 capsid antibody. At 42 days post-vaccination, one group of vaccinated and one group of mock-vaccinated animals were challenged with the predominant field strain PCV2b currently circulating in swine herds worldwide. Similarly, one vaccinated group and one mock-vaccinated group of pigs were challenged with the emerging PCV2d-2 virus. Blood samples were taken weekly after challenge and all animals were necropsied at 20 dpc.

As expected, pigs in the two vaccinated groups started to seroconvert to PCV2 capsid antibody by 42 dpv, whereas mock-vaccinated groups did not seroconvert until 7-14 dpc with PCV2b or PCV2d-2 (or 49 or 56 dpv, **Table 2**, **Fig. 4**). A qPCR assay targeting the PCV1 replicase gene (ORF1) was used to test for PCV1-3cl14 viral DNA from weekly sera, but PCV1-3cl14 viral DNA was undetectable and below the detection limit of the assay in any group after vaccination (data not shown). This is consistent with previous reports of the attenuated chimeric PCV1-2 virus infections in pigs (12, 37).

Only 2 out of 8 animals vaccinated and subsequently challenged with PCV2b had detectable viremia, and only at 14 dpc, compared to 4 and 7 out of 8 PCV2b challenge control animals at 14 and 20 dpc, respectively (**Table 2**). This difference was statistically significant, as the vaccinated and PCV2b challenged group had significantly lower levels of viral DNA loads in sera at 20 dpc, compared to mock-vaccinated and PCV2b challenged animals ($p<0.01$) (**Fig. 5**). For animals vaccinated and subsequently challenged with PCV2d-2, 1/8 at 14 dpc and 2/8 at 20 dpc had detectable viremia, while 7/8 PCV2d-2 challenged control animals were positive for serum viral DNA at 14 dpc and 20 dpc (**Table 2**). Also, the vaccinated and PCV2d-2 challenged group had serum viral DNA loads that were significantly reduced at 14 and 20 dpc ($p<0.001$, $p<0.05$, respectively), as compared to PCV2d-2 challenge only controls (**Fig. 5**). All vaccinated and subsequently challenged groups had significantly lower levels of PCV2 viremia at the peak of virus replication compared to control groups. In addition, all vaccinated and subsequently challenged groups had significantly lower levels of detectable PCV2 DNA in lymph nodes compared to mock-vaccinated and challenged groups (PCV2b = $p<0.001$, PCV2d-2 = $p<0.0001$, **Fig. 6**). These results indicated that vaccination with PCV1-3cl14 chimeric virus significantly reduces the level of virus replication in pigs when challenged with the predominant PCV2b subtype or with an emerging PCV2d-2 strain.

In addition to reducing viral DNA loads in sera and lymphoid tissues, vaccinated animals also had a decreased PCVAD lesion score compared to unvaccinated animals (**Fig. 7**). Vaccinated pigs that were subsequently challenged with PCV2b had significantly reduced pathological lesion scores for all measures of PCVAD, which includes lymphoid depletion and histiocytic replacement in lymph nodes, spleen, and tonsil tissues, as compared to unvaccinated but PCV2b challenged controls (**Fig. 7**). Similarly, pigs vaccinated and subsequently challenged with PCV2d-2 had significantly lower pathological lesion scores for lymph node measures, as well as tonsil lymphoid depletion (**Fig. 8A, 8B, 8E**) as compared to unvaccinated but PCV2d-2 challenged controls. Consistent with the results for serum and lymph node viral DNA detection, both vaccinated and subsequently challenged groups had significantly lower viral antigen scores in lymph node, spleen, and tonsil, compared to challenge only controls (**Fig. 8**). Overall, these results suggest that vaccination with PCV1-3cl14 chimeric virus vaccine candidate protects against two genetically distinct and relevant PCV2 strains, the predominant PCV2b subtype currently circulating in pig farms worldwide and the emerging PCV2d-2 strain.

Discussion

PCVAD is arguably one of the most economically-important diseases affecting the global swine industry. Characterized by progressive wasting, hallmark histological lesions of lymphoid depletion with histiocytic infiltration, and the presence of PCV2 antigen or DNA in the lesions, PCVAD is caused by PCV2 infection, although co-infection with other pathogens are usually necessary for the development of the full-spectrum of clinical PCVAD (44-46). Several commercial vaccines against PCV2 are currently available, all of which are based on the PCV2a subtype (11), which prior to 2005 was the main subtype (15-17). However, now PCV2b has surpassed PCV2a as the most prevalent strain associated with PCVAD losses in the swine industry (15-17). ~~In addition, recently, speculation of vaccination failures has been reported, and though no direct evidence has been found as of yet, these~~ A~~nd~~lthough all current vaccines have been proven effective at preventing clinical signs and global economic loss due to PCVAD, ~~events have been associated with~~ the emergence of the PCV2d (or mutant PCV2b) subtype (18, 19, 24), as well as the replacement of PCV2a with PCV2b as the predominant circulating subtype, cannot be ignored. ~~(18, 19, 24).~~ Therefore, it is logical to develop the next generation of vaccines especially against the emerging PCV2 strains.

The objectives of this study were to molecularly breed the capsid genes from different PCV2 subtypes by DNA shuffling, and to develop a candidate chimeric virus vaccine based on the non-pathogenic PCV1 backbone and shuffled capsid genes of divergent PCV2 subtypes. Traditional DNA shuffling approach was undertaken in this study, in which 5 genetically distinct capsid sequences from each of the 4 known PCV2 subtypes, as well as from the "divergent PCV2a~~PCV2e~~" type (47), which is now generally considered as a divergent PCV2a strain (35), were used for the DNA shuffling. Of the more than 50 shuffled PCV2 capsids that were cloned and sequenced, infectious chimeric viruses were rescued in PK15 cells only in 4 of them, suggesting that the small PCV2 genome cannot support a large number of forced random reassortment within the capsid gene.

The four viable viruses with shuffled PCV2 capsids generated by traditional DNA shuffling contained antigenic epitopes from all 5 genetically divergent PCV2 strains, although most of the variability in the shuffled capsids could be found in the PCV2c parental strain. This was not unexpected, as the PCV2c subtype is the most divergent strain from the rest of the PCV2 subtypes identified thus far, based on a phylogenetic analysis (24, 48). Alignment of the 5 selected parental strains revealed that the PCV2c does, in fact, contain the most genetically distinct amino acid variations, though some of these amino acids overlap with the parental PCV2d strain, including the addition of a terminal lysine residue. The presence of amino acid residues unique to PCV2c and PCV2d strains suggests that, although the PCV2c subtype has not associated with any clinical disease, this subtype could possibly have contributed to the evolutionary emergence of the current PCV2d subtype. In fact, the PCV2c subtype was recently isolated from feral pigs in Brazil for first time since it was originally described in Denmark in the early 90s. The feral pig populations were also infected with the other three PCV2 subtypes, suggesting the possibility of recombination (23). Therefore, these findings support the inclusion of PCV2c for DNA shuffling in the current study in order to increase the breadth of protection of the resulting candidate vaccine against currently emerging and future possible emerging PCV2 strains.

In order to determine the *in vivo* infectivity of the shuffled viruses and to screen for the best chimera for subsequent challenge and efficacy study, conventional pigs were experimentally inoculated in a pilot study with each of the 4 viruses with shuffled capsids in the PCV2a backbone as well as with the chimeric PCV1-2a vaccine virus (12). The results showed that virus PCV2-3cl.14 induced higher levels of neutralizing antibody titers when compared to the chimeric PCV1-2a virus, as well as the other 3 shuffled capsid viruses. The chimeric virus PCV2-3cl.14 also induced significantly higher neutralizing antibody titers against PCV2a and PCV2d-2 strains. The fact that the PCV2-3cl1.14 shuffled capsid virus induced higher neutralizing antibody titers against PCV2a compared to a homologous vaccination with the PCV1-2a chimeric vaccine strain was unexpected. However others have demonstrated this phenomenon with PCV2 viruses before. Although they demonstrate opposing results, there are many differences in the experimental design, which could explain these ~~discrepencies~~discrepancies (21, 49). In addition, the PCV2-3cl14 virus strain grew to the lowest titer of 10^3.33 $TCID_{50}$/mL compared to the other PCV2-shuffled capsid strains and the PCV1-2a vaccine strain *in vitro* on multiple occasions (data not shown), suggesting that the increase in total and breadth of neutralizing antibody titers compared to the other strains tested was not simply due to increased replication efficiency. Taken together, these results demonstrate that more research is needed to understand the complicated nature of PCV2 capsid immunogenicity. Comparison of the amino acid sequences of the shuffled capsid 3cl14

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to the other three shuffled capsids as well as the PCV1-2a reveals three regions with distinct amino acid residues. Two of these regions, amino acids 106-108 and 126, overlap with previously-identified B-cell antigenic epitopes (50). In addition, the mutation at position 126 corresponded to a location within the predicted B-cell and SLA-class II epitopes (51). It is also possible that mutations within regions unrecognized as immunogenic may play a direct role in the protective immune response, or alter structural recognition of other immunogenic capsid regions, such as the 169-180 region shown to play a "decoy" role in anti-PCV2 antibody recognition (52, 53). While the 3cl14 residues at 169-180 are identical to the strain used to demonstrate the decoy nature of this region, changes at other locations may result exposure of this region to antibody neutralization. While the majority of PCV2c amino acid residues introduced into the 3cl14 shuffled capsid residues that map to a subset of the parental strains, but not one distinct subtype have been introduced. The 3cl14 sequence contains amino acids at positions 14 and 232 that represent the PCV2a and "divergent PCV2a" as well as an amino acid residue that is shared by PCV2d, PCV2b, and PCV2, but not divergent PCV2a or PCV2c at position 21, and a residue shared by the parental PCV2b and PCV2d but not PCV2c, PCV2a, or "divergent PCV2a" at position 185. Interestingly, the 3cl13, 3cl4_2, and 3cl12_2 shuffled capsids all contain the additional lysine residue at the C-terminus of the capsid found in the PCV2d parental strain. This mutation is suggested to play a role in the increased pathogenicity and vaccine failure of the emerging PCV2d strains, although no direct evidence of this role has been reported to date (38, 54). However, the 3cl14 shuffled capsid sequence does not include the additional lysine, suggesting that it is not a necessary epitope for producing neutralizing antibodies against the PCV2d-2 strains,

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Formatted: Font: (Default) Times New Roman, 12 pt, Not Italic since PCV1-3cl14 protects against PCV2d-2 infection in the challenge and efficacy experiment. While it is possible that the properties of 3cl14 capsid sequence discussed above are important for production of cross-protective neutralizing antibodies in pigs, additional research is warranted to determine the important amino acid residues that may play a critical role in conferring cross-neutralizing activities against different PCV2 subtypes.

Based on induction of significantly higher cross-neutralizing antibody titers, compared to the other shuffled capsid candidates, the shuffled 3cl14 capsid sequence was subsequently selected to produce a chimeric virus PCV1-3cl14 vaccine candidate. The protective efficacy of the PCV1-3cl14 chimeric virus as a potential vaccine was evaluated by challenging vaccinated pigs with PCV2b or PCV2d, respectively. PCV2b is the predominant subtype currently infecting pigs worldwide, whereas the PCV2d is an emerging subtype (24). We previously have demonstrated the attenuation of chimeric PCV1-2a and PCV1-2b viruses in the genomic backbone of the non-pathogenic PCV1 *in vivo* (12, 37, 39). Consistent with these previous reports, there was no detectable PCV1-3cl14 viremia in vaccinated pigs throughout the duration of the study, and no detectable clinical disease prior to challenge with either PCV2b or PCV2d (data not shown), even though the vaccinated pigs are infected as evidenced by seroconversion to PCV2 capsid antibody. It is also possible that the standard PCV2a capsid-based PCV2 ORF2 ELISA assay is less sensitive for detection of the PCV1-shuffle capsid induced antibodies, possibly leading an underrepresentation of the antibody titers in the PCV1-3cl14 vaccinated groups, however further research is needed to determine if this is the case. Whether the serology data is indeed blunted due to the limitations of the assay, the reduction in challenge virus levels shows a significant effect of vaccination with the PCV1-3cl14 vaccine candidate on PCV2b and PCV2d challenge strains.

Vaccination with the chimeric virus PCV1-3cl14 vaccine candidate resulted in significantly reduced PCV2b or PCV2d viral DNA loads at the peak of viremia as well as reduced viral DNA loads in lymphoid tissues at termination of the study. Furthermore, the lymphoid lesions were also significantly reduced in vaccinated groups subsequently challenged with PCV2b compared to mock-vaccinated and challenged controls. Though the vaccinated animals showed no statistically significant reduction in spleen lymphoid depletion and spleen and tonsil hystiocytic replacement when challenged with PCV2d, they did have significant reduction for the rest of the PCVAD-associated scores, as well as reduced viral DNA loads in serum and lymph node tissues, indicating that the PCV1-3cl14 chimeric virus vaccine candidate induced protection against both PCV2b and PCV2d challenge in conventional pigs.

Conclusion

To our knowledge, this is the first report of construction of viable chimeric PCV2 vaccine candidate by shuffling the capsid gene of 5 divergent PCV2 strains belonging to different subtypes. Importantly, vaccination of pigs with a chimeric virus PCV1-3cl14 with shuffled capsid genes induced protective immunity against challenge with the predominant PCV2b subtype and the emerging PCV2d subtype. Therefore, this chimeric virus is a potential candidate for further development into the next generation of vaccine against PCV2.

Conflict of Interest Declaration

X.J. Meng is the lead inventor of the chimeric PCV1-2a upon which the current commercial vaccines Fostera™ PCV and Fostera™ PCV MH are based. Greg Nitzel and David Slade are both employees of Zoetis Inc.

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References

1. **Todd D BM, Biagini P, Hino S, Mankertz A, Mishiro S, Niel C, Okamoto H, Raidal S, Ritchie BW, Teo GC.** 2005. Circoviridae. Press EA, San Diego.
2. **Allan GM, McNeilly F, Kennedy S, Daft B, Clarke EG, Ellis JA, Haines DM, Meehan BM, Adair BM.** 1998. Isolation of porcine circovirus-like viruses from pigs with a wasting disease in the USA and Europe. J Vet Diagn Invest **10:**3-10.
3. **Tischer I, Mields W, Wolff D, Vagt M, Griem W.** 1986. Studies on epidemiology and pathogenicity of porcine circovirus. Arch Virol **91:**271-276.
4. **Meehan BM, McNeilly F, Todd D, Kennedy S, Jewhurst VA, Ellis JA, Hassard LE, Clark EG, Haines DM, Allan GM.** 1998. Characterization of novel circovirus DNAs associated with wasting syndromes in pigs. J Gen Virol **79 (Pt 9):**2171-2179.
5. **Ellis J, Hassard L, Clark E, Harding J, Allan G, Willson P, Strokappe J, Martin K, McNeilly F, Meehan B, Todd D, Haines D.** 1998. Isolation of circovirus from lesions of pigs with postweaning multisystemic wasting syndrome. Can Vet J **39:**44-51.
6. **Choi C, Chae C, Clark EG.** 2000. Porcine postweaning multisystemic wasting syndrome in Korean pig: detection of porcine circovirus 2 infection by immunohistochemistry and polymerase chain reaction. J Vet Diagn Invest **12:**151-153.
7. **Edwards S, Sands JJ.** 1994. Evidence of circovirus infection in British pigs. Vet Rec **134:**680-681.
8. **Larochelle R, Morin M, Antaya M, Magar R.** 1999. Identification and incidence of porcine circovirus in routine field cases in Quebec as determined by PCR. Vet Rec **145:**140-142.
9. **Onuki A, Abe K, Togashi K, Kawashima K, Taneichi A, Tsunemitsu H.** 1999. Detection of porcine circovirus from lesions of a pig with wasting disease in Japan. J Vet Med Sci **61:**1119-1123.
10. **Opriessnig T, Meng XJ, Halbur PG.** 2007. Porcine circovirus type 2 associated disease: update on current terminology, clinical manifestations, pathogenesis, diagnosis, and intervention strategies. J Vet Diagn Invest **19:**591-615.
11. **Beach NM, Meng XJ.** 2012. Efficacy and future prospects of commercially available and experimental vaccines against porcine circovirus type 2 (PCV2). Virus Res **164:**33-42.
12. **Fenaux M, Opriessnig T, Halbur PG, Elvinger F, Meng XJ.** 2004. A chimeric porcine circovirus (PCV) with the immunogenic capsid gene of the pathogenic PCV type 2 (PCV2) cloned into the genomic backbone of the nonpathogenic PCV1 induces protective immunity against PCV2 infection in pigs. J Virol **78:**6297-6303.
13. **Opriessnig T, Madson DM, Prickett JR, Kuhar D, Lunney JK, Elsener J, Halbur PG.** 2008. Effect of porcine circovirus type 2 (PCV2) vaccination on

porcine reproductive and respiratory syndrome virus (PRRSV) and PCV2 coinfection. Vet Microbiol **131:**103-114.

14. **Chae C.** 2012. Commercial porcine circovirus type 2 vaccines: efficacy and clinical application. Vet J **194:**151-157.
15. **Cheung AK, Lager KM, Kohutyuk OI, Vincent AL, Henry SC, Baker RB, Rowland RR, Dunham AG.** 2007. Detection of two porcine circovirus type 2 genotypic groups in United States swine herds. Arch Virol **152:**1035-1044.
16. **Gagnon CA, Tremblay D, Tijssen P, Venne MH, Houde A, Elahi SM.** 2007. The emergence of porcine circovirus 2b genotype (PCV-2b) in swine in Canada. Can Vet J **48:**811-819.
17. **Firth C, Charleston MA, Duffy S, Shapiro B, Holmes EC.** 2009. Insights into the evolutionary history of an emerging livestock pathogen: porcine circovirus 2. J Virol **83:**12813-12821.
18. **Opriessnig T, Xiao CT, Gerber PF, Halbur PG.** 2013. Emergence of a novel mutant PCV2b variant associated with clinical PCVAD in two vaccinated pig farms in the U.S. concurrently infected with PPV2. Vet Microbiol **163:**177-183.
19. **Seo HW, Park C, Kang I, Choi K, Jeong J, Park SJ, Chae C.** 2014. Genetic and antigenic characterization of a newly emerging porcine circovirus type 2b mutant first isolated in cases of vaccine failure in Korea. Arch Virol **159:**3107-3111.
20. **Eddicks M, Fux R, Szikora F, Eddicks L, Majzoub-Altweck M, Hermanns W, Sutter G, Palzer A, Banholzer E, Ritzmann M.** 2015. Detection of a new cluster of porcine circovirus type 2b strains in domestic pigs in Germany. Vet Microbiol **176:**337-343.
21. **Huang L, Wang Y, Wei Y, Chen D, Liu D, Du W, Xia D, Wu H, Feng L, Liu C.** 2016. Capsid proteins from PCV2a genotype confer greater protection against a PCV2b strain than those from PCV2b genotype in pigs: evidence for PCV2b strains becoming more predominant than PCV2a strains from 2000 to 2010s. Appl Microbiol Biotechnol doi:10.1007/s00253-016-7459-y.
22. **Allan G, McNeilly F, Meehan B, McNair I, Ellis J, Krakowka S, Fossum C, Wattrang E, Wallgren P, Adair B.** 2003. Reproduction of postweaning multisystemic wasting syndrome in pigs experimentally inoculated with a Swedish porcine circovirus 2 isolate. J Vet Diagn Invest **15:**553-560.
23. **Franzo G, Cortey M, de Castro AM, Piovezan U, Szabo MP, Drigo M, Segales J, Richtzenhain LJ.** 2015. Genetic characterisation of Porcine circovirus type 2 (PCV2) strains from feral pigs in the Brazilian Pantanal: An opportunity to reconstruct the history of PCV2 evolution. Vet Microbiol **178:**158-162.
24. **Xiao CT, Halbur PG, Opriessnig T.** 2015. Global molecular genetic analysis of porcine circovirus type 2 (PCV2) sequences confirms the presence of four main PCV2 genotypes and reveals a rapid increase of PCV2d. J Gen Virol **96:**1830-1841.
25. **Dvorak CM, Yang Y, Haley C, Sharma N, Murtaugh MP.** 2016. National reduction in porcine circovirus type 2 prevalence following introduction of vaccination. Vet Microbiol **189:**86-90.

26. **Saha D, Huang L, Bussalleu E, Lefebvre DJ, Fort M, Van Doorsselaere J, Nauwynck HJ.** 2012. Antigenic subtyping and epitopes' competition analysis of porcine circovirus type 2 using monoclonal antibodies. Vet Microbiol **157:**13-22.
27. **Saha D, Lefebvre DJ, Ooms K, Huang L, Delputte PL, Van Doorsselaere J, Nauwynck HJ.** 2012. Single amino acid mutations in the capsid switch the neutralization phenotype of porcine circovirus 2. J Gen Virol **93:**1548-1555.
28. **Stemmer WP.** 1994. DNA shuffling by random fragmentation and reassembly: in vitro recombination for molecular evolution. Proc Natl Acad Sci U S A **91:**10747-10751.
29. **Stemmer WP.** 1994. Rapid evolution of a protein in vitro by DNA shuffling. Nature **370:**389-391.
30. **Zhou L, Ni YY, Pineyro P, Sanford BJ, Cossaboom CM, Dryman BA, Huang YW, Cao DJ, Meng XJ.** 2012. DNA shuffling of the GP3 genes of porcine reproductive and respiratory syndrome virus (PRRSV) produces a chimeric virus with an improved cross-neutralizing ability against a heterologous PRRSV strain. Virology **434:**96-109.
31. **Ni YY, Opriessnig T, Zhou L, Cao D, Huang YW, Halbur PG, Meng XJ.** 2013. Attenuation of porcine reproductive and respiratory syndrome virus by molecular breeding of virus envelope genes from genetically divergent strains. J Virol **87:**304-313.
32. **Tian D, Ni YY, Zhou L, Opriessnig T, Cao D, Pineyro P, Yugo DM, Overend C, Cao Q, Lynn Heffron C, Halbur PG, Pearce DS, Calvert JG, Meng XJ.** 2015. Chimeric porcine reproductive and respiratory syndrome virus containing shuffled multiple envelope genes confers cross-protection in pigs. Virology **485:**402-413.
33. **Zhou L, Ni YY, Pineyro P, Cossaboom CM, Subramaniam S, Sanford BJ, Dryman BA, Huang YW, Meng XJ.** 2013. Broadening the heterologous cross-neutralizing antibody inducing ability of porcine reproductive and respiratory syndrome virus by breeding the GP4 or M genes. PLoS One **8:**e66645.
34. **Wang F, Guo X, Ge X, Wang Z, Chen Y, Cha Z, Yang H.** 2009. Genetic variation analysis of Chinese strains of porcine circovirus type 2. Virus Res **145:**151-156.
35. **Cortey M, Olvera A, Grau-Roma L, Segales J.** 2011. Further comments on porcine circovirus type 2 (PCV2) genotype definition and nomenclature. Vet Microbiol **149:**522-523.
36. **Fenaux M, Halbur PG, Haqshenas G, Royer R, Thomas P, Nawagitgul P, Gill M, Toth TE, Meng XJ.** 2002. Cloned genomic DNA of type 2 porcine circovirus is infectious when injected directly into the liver and lymph nodes of pigs: characterization of clinical disease, virus distribution, and pathologic lesions. J Virol **76:**541-551.
37. **Beach NM, Ramamoorthy S, Opriessnig T, Wu SQ, Meng XJ.** 2010. Novel chimeric porcine circovirus (PCV) with the capsid gene of the emerging PCV2b subtype cloned in the genomic backbone of the non-pathogenic PCV1

is attenuated in vivo and induces protective and cross-protective immunity against PCV2b and PCV2a subtypes in pigs. Vaccine **29:**221-232.

38. **Opriessnig T, Xiao CT, Gerber PF, Halbur PG, Matzinger SR, Meng XJ.** 2014. Mutant USA strain of porcine circovirus type 2 (mPCV2) exhibits similar virulence to the classical PCV2a and PCV2b strains in caesarean-derived, colostrum-deprived pigs. J Gen Virol **95:**2495-2503.

39. **Fenaux M, Opriessnig T, Halbur PG, Meng XJ.** 2003. Immunogenicity and pathogenicity of chimeric infectious DNA clones of pathogenic porcine circovirus type 2 (PCV2) and nonpathogenic PCV1 in weanling pigs. J Virol **77:**11232-11243.

40. **Opriessnig T, Thacker EL, Yu S, Fenaux M, Meng XJ, Halbur PG.** 2004. Experimental reproduction of postweaning multisystemic wasting syndrome in pigs by dual infection with Mycoplasma hyopneumoniae and porcine circovirus type 2. Vet Pathol **41:**624-640.

41. **Halbur PG, Paul PS, Frey ML, Landgraf J, Eernisse K, Meng XJ, Lum MA, Andrews JJ, Rathje JA.** 1995. Comparison of the pathogenicity of two US porcine reproductive and respiratory syndrome virus isolates with that of the Lelystad virus. Vet Pathol **32:**648-660.

42. **Beach NM, Smith SM, Ramamoorthy S, Meng XJ.** 2011. Chimeric porcine circoviruses (PCV) containing amino acid epitope tags in the C terminus of the capsid gene are infectious and elicit both anti-epitope tag antibodies and anti-PCV type 2 neutralizing antibodies in pigs. J Virol **85:**4591-4595.

43. **Nawagitgul P, Harms PA, Morozov I, Thacker BJ, Sorden SD, Lekcharoensuk C, Paul PS.** 2002. Modified indirect porcine circovirus (PCV) type 2-based and recombinant capsid protein (ORF2)-based enzyme-linked immunosorbent assays for detection of antibodies to PCV. Clin Diagn Lab Immunol **9:**33-40.

44. **Segales J, Allan GM, Domingo M.** 2005. Porcine circovirus diseases. Anim Health Res Rev **6:**119-142.

45. **Albina E, Truong C, Hutet E, Blanchard P, Cariolet R, L'Hospitalier R, Mahe D, Allee C, Morvan H, Amenna N, Le Dimna M, Madec F, Jestin A.** 2001. An experimental model for post-weaning multisystemic wasting syndrome (PMWS) in growing piglets. J Comp Pathol **125:**292-303.

46. **Magar R, Larochelle R, Thibault S, Lamontagne L.** 2000. Experimental transmission of porcine circovirus type 2 (PCV2) in weaned pigs: a sequential study. J Comp Pathol **123:**258-269.

47. **Jantafong T, Boonsoongnern A, Poolperm P, Urairong K, Lekcharoensuk C, Lekcharoensuk P.** 2011. Genetic characterization of porcine circovirus type 2 in piglets from PMWS-affected and -negative farms in Thailand. Virol J **8:**88.

48. **Franzo G, Cortey M, Olvera A, Novosel D, De Castro AM, Biagini P, Segales J, Drigo M.** 2015. Revisiting the taxonomical classification of Porcine Circovirus type 2 (PCV2): still a real challenge. Virol J **12:**131.

49. **Opriessnig T, O'Neill K, Gerber PF, de Castro AM, Gimenez-Lirola LG, Beach NM, Zhou L, Meng XJ, Wang C, Halbur PG.** 2013. A PCV2 vaccine based on genotype 2b is more effective than a 2a-based vaccine to protect

against PCV2b or combined PCV2a/2b viremia in pigs with concurrent PCV2, PRRSV and PPV infection. Vaccine **31:**487-494.

50. **Ge M, Yan A, Luo W, Hu YF, Li RC, Jiang DL, Yu XL.** 2013. Epitope screening of the PCV2 Cap protein by use of a random peptide-displayed library and polyclonal antibody. Virus Res **177:**103-107.

51. **Constans M, Ssemadaali M, Kolyvushko O, Ramamoorthy S.** 2015. Antigenic Determinants of Possible Vaccine Escape by Porcine Circovirus Subtype 2b Viruses. Bioinform Biol Insights **9:**1-12.

52. **Trible BR, Kerrigan M, Crossland N, Potter M, Faaberg K, Hesse R, Rowland RR.** 2011. Antibody recognition of porcine circovirus type 2 capsid protein epitopes after vaccination, infection, and disease. Clin Vaccine Immunol **18:**749-757.

53. **Trible BR, Ramirez A, Suddith A, Fuller A, Kerrigan M, Hesse R, Nietfeld J, Guo B, Thacker E, Rowland RR.** 2012. Antibody responses following vaccination versus infection in a porcine circovirus-type 2 (PCV2) disease model show distinct differences in virus neutralization and epitope recognition. Vaccine **30:**4079-4085.

54. **Opriessnig T, Gerber PF, Xiao CT, Halbur PG, Matzinger SR, Meng XJ.** 2014. Commercial PCV2a-based vaccines are effective in protecting naturally PCV2b-infected finisher pigs against experimental challenge with a 2012 mutant PCV2. Vaccine **32:**4342-4348.

Figure Legends

Fig. 1. Amino acid sequence alignment of the capsid proteins from the five parental PCV2 wild-type strains and the four candidate DNA-shuffled capsids evaluated in this study. The first five sequences represent the parental strains including PCV2a (strain 40895, GenBank accession number AF264042), PCV2b (strain NC16845, accession number GU799576), PCV2c (accession number EU148503), PCV2d (accession number AY181947), and "divergent PCV2a" (accession number EF524533). while the bottom four sequences represent the DNA-shuffled PCV2 capsids. Amino acids that differ from the consensus are shown in black.

Fig. 2. A phylogenetic tree of the capsid genes of selected PCV2 strains from different subtypes. The phylogenetic tree was constructed using the neighbor-joining method with bootstraps in 1,000 replicates. The number above each major branch indicates the bootstrap value. The bold italicized sequence names represent the PCV2 sequences of the 5 parental strains used for DNA shuffling in the study.

Fig. 3. Comparison of 50% neutralizing antibody titers against four PCV2 wild-type strains from sera of pigs experimentally inoculated with chimeric viruses PCV2-3cl13, PCV2-3cl14, PCV2-3cl4_2, and PCV2-3cl12_2, or PCV1-2a with shuffled capsid genes. *In vitro* 50% neutralization assay of respective sera collected at 56 days post-infection against three parental PCV2 strains: (**A**) PCV2a, (**B**) PCV2d-1, (**C**) PCV2b, and (**D**) PCV2d-2 isolate. The NA titers were calculated as the highest 2-fold dilution (2^n) of the serum sample that showed a 50% or greater reduction in the number

of positive fluorescent foci, compared to the serum samples from the mock (PBS) inoculated control group in the same dilution. Asterisk (*) sign indicates $p<0.05$ analyzed using one-way ANOVA.

Fig. 4. PCV2 capsid-specific antibody response in conventional pigs experimentally inoculated with the chimeric virus PCV1-3cl14 vaccine candidate and challenged with the wild-type virus strains PCV2b or PCV2d-2. The mean S/P ratio ± SEM is plotted for each treatment group throughout the duration of the study. The virus challenge took place at 42 days post-vaccination (dpv). The dashed line at 0.2 S/P ratio denotes the lower end cutoff for a positive sample in this assay.

Fig. 5. Quantification of PCV2 viral DNA loads in sera from pigs vaccinated with the chimeric PCV1-3cl14 virus and subsequently challenged with PCV2b or PCV2d-2 compared to challenge only controls. Quantification of PCV2 ORF1 viral DNA loads in sera using qPCR in (**A**) PCV2b challenged and (**B**) PCV2d-2 challenged animals. Group means ± SEM are plotted for each time point post-challenge. The limit of detection for the assay was $10^{4.2}$ copies/mL serum of ORF1 DNA determined by a standard curve for 10^1 – 10^{10} copies of the wild-type PCV2b genome. (*) Indicates statistical significance between groups (Student's t-test, corrected for multiple tests).

Fig. 6: Quantification of PCV2 viral DNA loads in lymph nodes from pigs vaccinated with the chimeric PCV1-3cl14 virus and challenged with PCV2b or PCV2d-2 compared to challenge only controls. Quantification of PCV2 ORF1 viral

DNA loads in lymph nodes using qPCR in (**A**) PCV2b challenged (**B**) and PCV2d-2 challenged animals. Group means ± SEM are plotted for each time point post-challenge. The limit of detection for the assay was $10^{7.1}$ copies/mg tissue of ORF1 viral DNA, as determined by a standard curve for $10^{1} – 10^{10}$ copies of the wild-type PCV2b genome. (*) Indicates statistical significance between groups at that time point (Student's t-test, corrected for multiple tests).

Fig. 7. Comparison of lymphoid tissues in pigs vaccinated with the chimeric PCV1-3cl.14 virus and subsequently challenged with PCV2b or PCV2d-2 with those of challenge only controls. Lymphoid depletion and histiocytic replacement for (**A, B**) lymph nodes, (**C, D**) spleen, and (**E, F**) tonsils at necropsy were compared for vaccinated and challenged animals (■) with those of challenge only controls (**O**). Individual animal scores are represented by individual symbols and group means ± SEM are displayed. Asterisk (*) sign indicates statistically significant differences between groups (student's t-test).

Fig. 8. Quantification of PCV2 viral antigen in lymphoid tissues by PCV2 immunohistochemistry (IHC). The tissues were obtained from pigs vaccinated with the chimeric PCV1-3cl.14 virus and subsequently challenged with PCV2b or PCV2d-2 compared to challenge only controls. PCV2 viral antigen scores determined for (**A**) lymph nodes, (**B**) spleen, and (**C**) tonsils at necropsy were compared for vaccinated and challenged animals (■) with those of challenge only controls (**O**). Individual animal scores are represented by individual symbols and group means ± SEM

are displayed. Asterisk (*) sign indicates statistically significant differences between groups (student's t-test).

Figures 1-8

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Figure 1

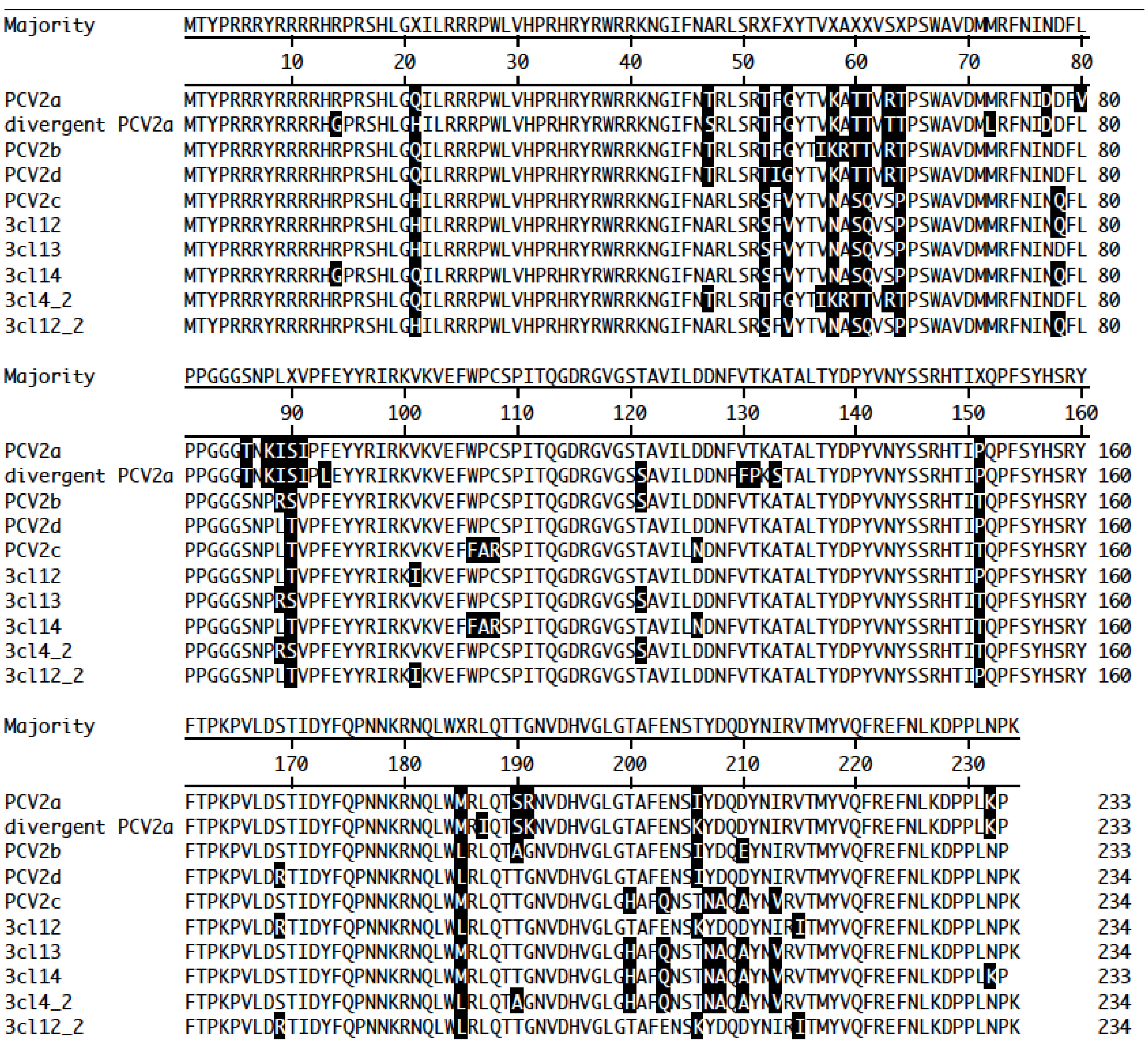
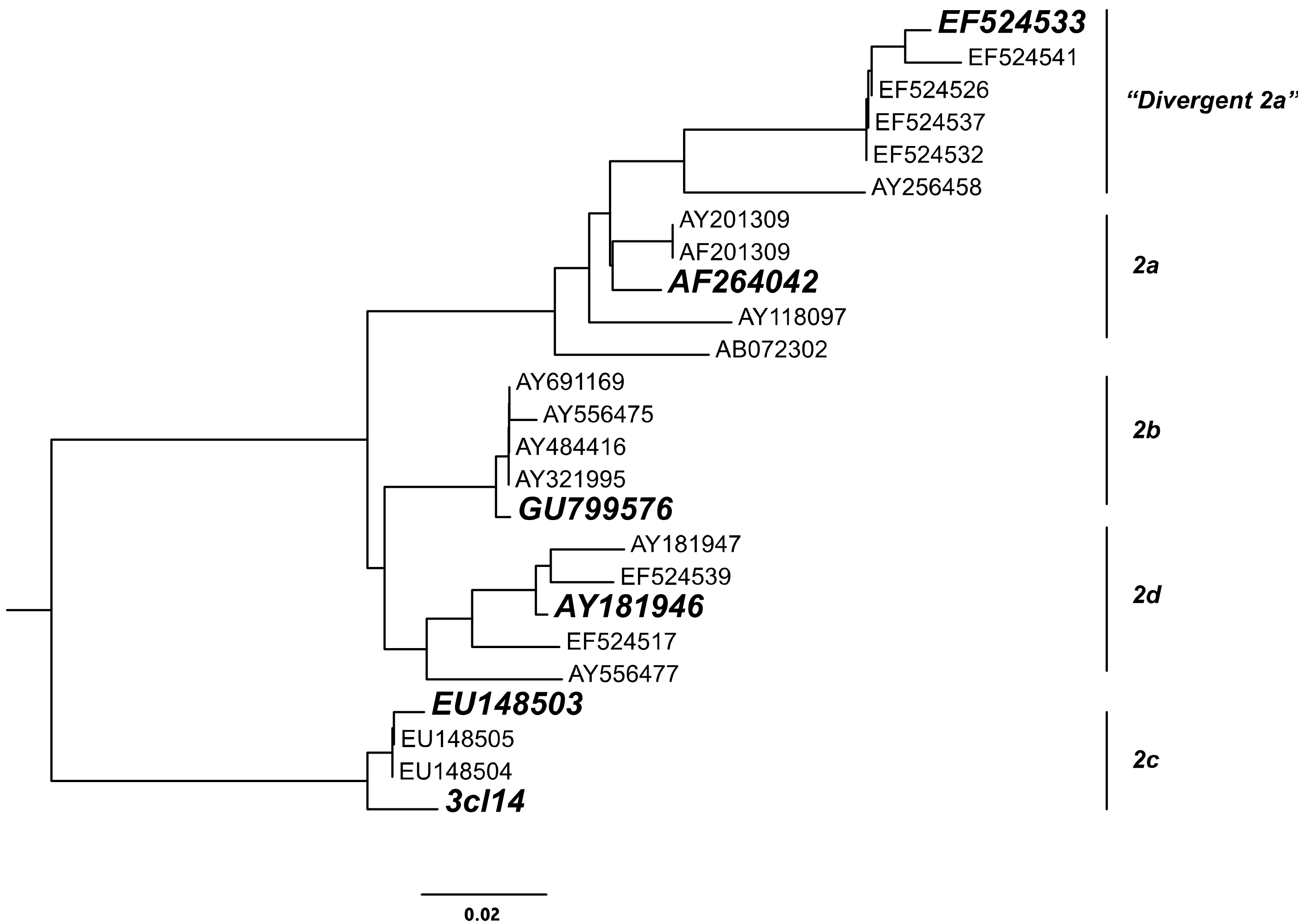


Figure 2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AY201309
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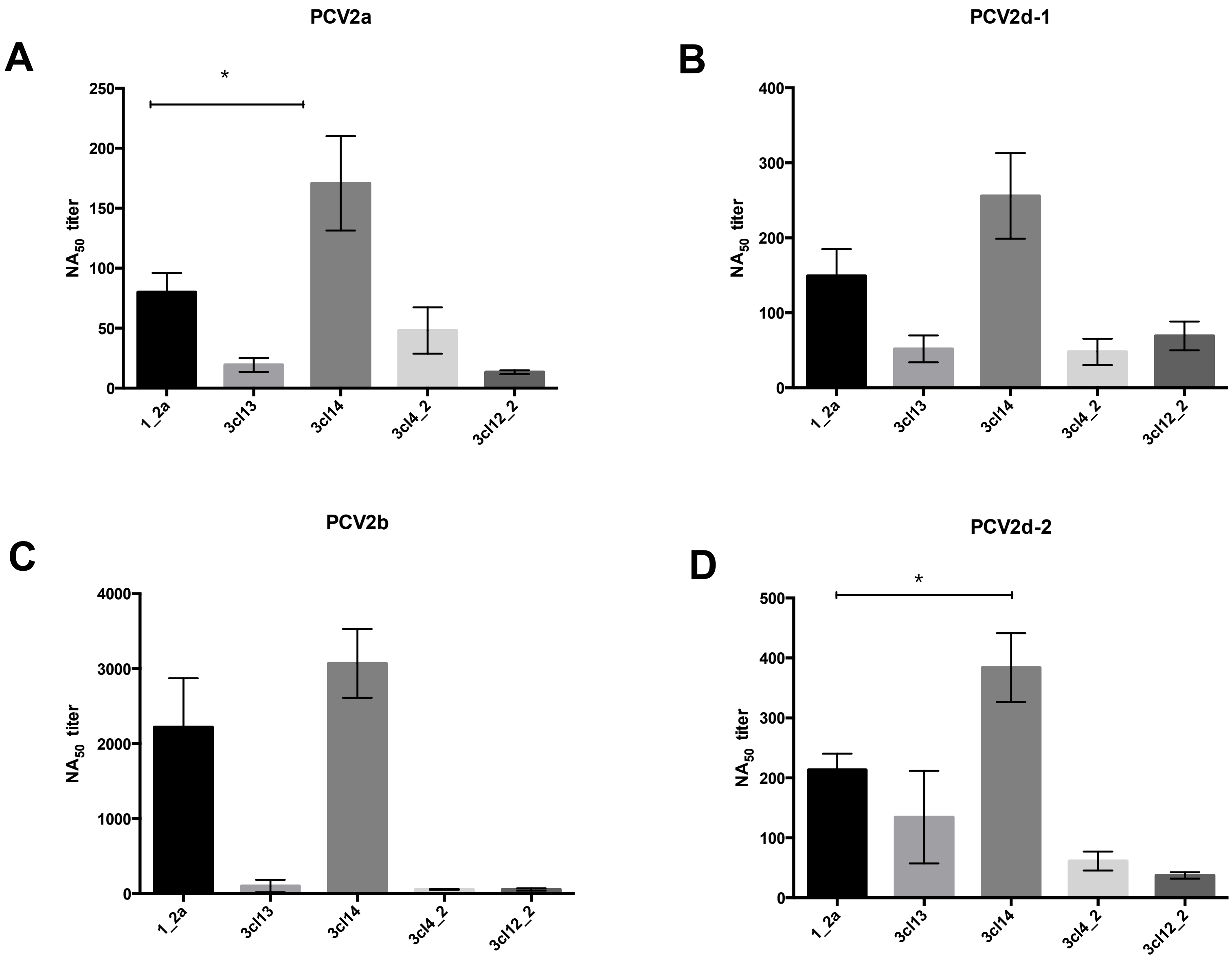
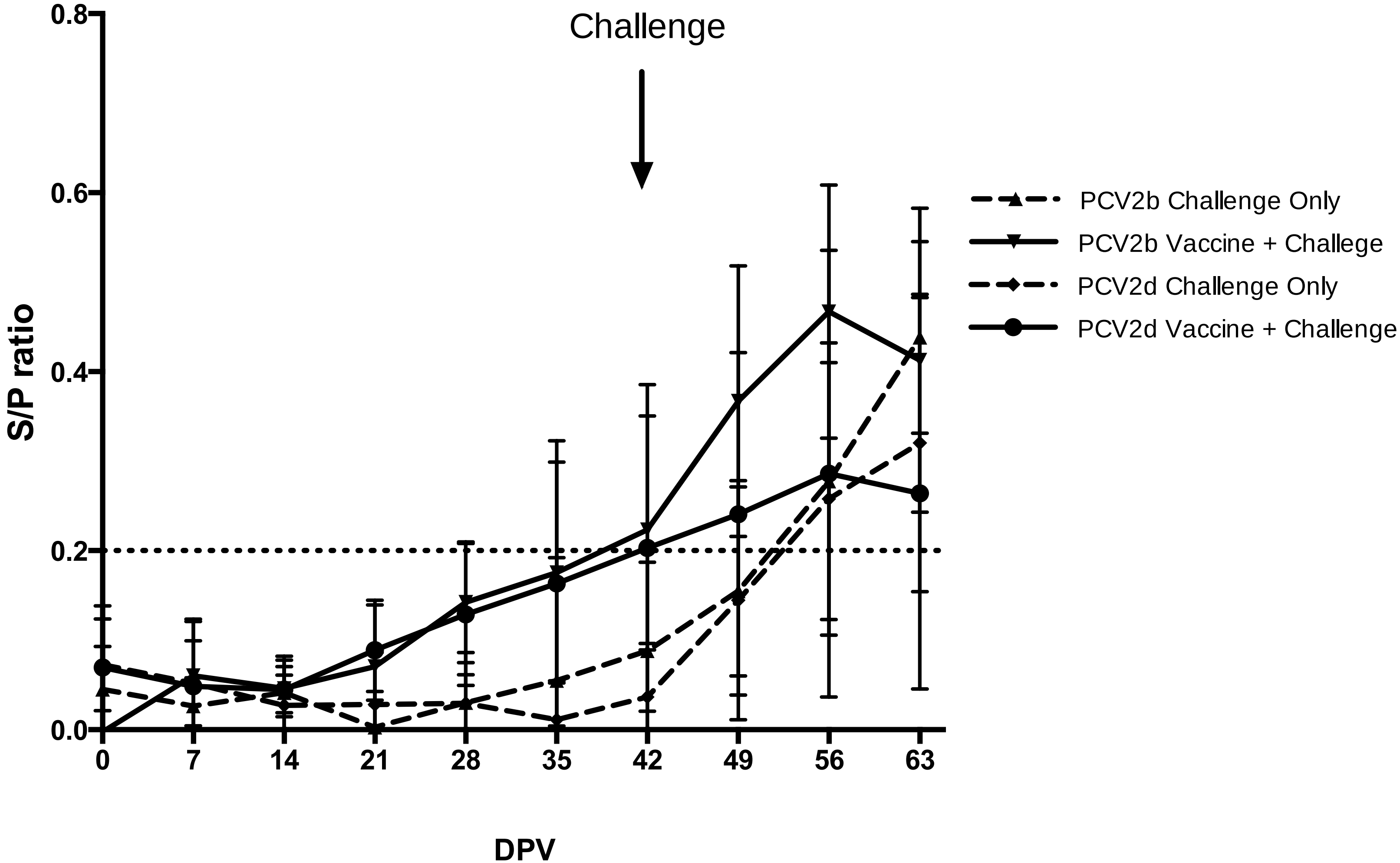
AB072302
2a
AY691169
AY556475
AY484416
AY321995
GU799576
2b
AY181947
EF524539
AY181946
EF524517
AY556477
2d
EU148503
EU148505
EU148504
3cl14
2c
0.02

Figure 3



PCV2a
A
*
250
200
150
100
50
0
NA50 titer
1_2a
3cl13
3cl14
3cl4_2
3cl12_2
PCV2d-1
B
400
300
200
100
0
NA50 titer
1_2a
3cl13
3cl14
3cl4_2
3cl12_2
PCV2b
C
4000
3000
2000
1000
0
NA50 titer
1_2a
3cl13
3cl14
3cl4_2
3cl12_2
PCV2d-2
D
*
500
400
300
200
100
0
NA50 titer
1_2a
3cl13
3cl14
3cl4_2
3cl12_2

Figure 4



Challenge
0.8
0.6
0.4
0.2
0.0
S/P ratio
0
7
14
21
28
35
42
49
56
63
DPV
PCV2b Challenge Only
PCV2b Vaccine + Challege
PCV2d Challenge Only
PCV2d Vaccine + Challenge

Figure 5

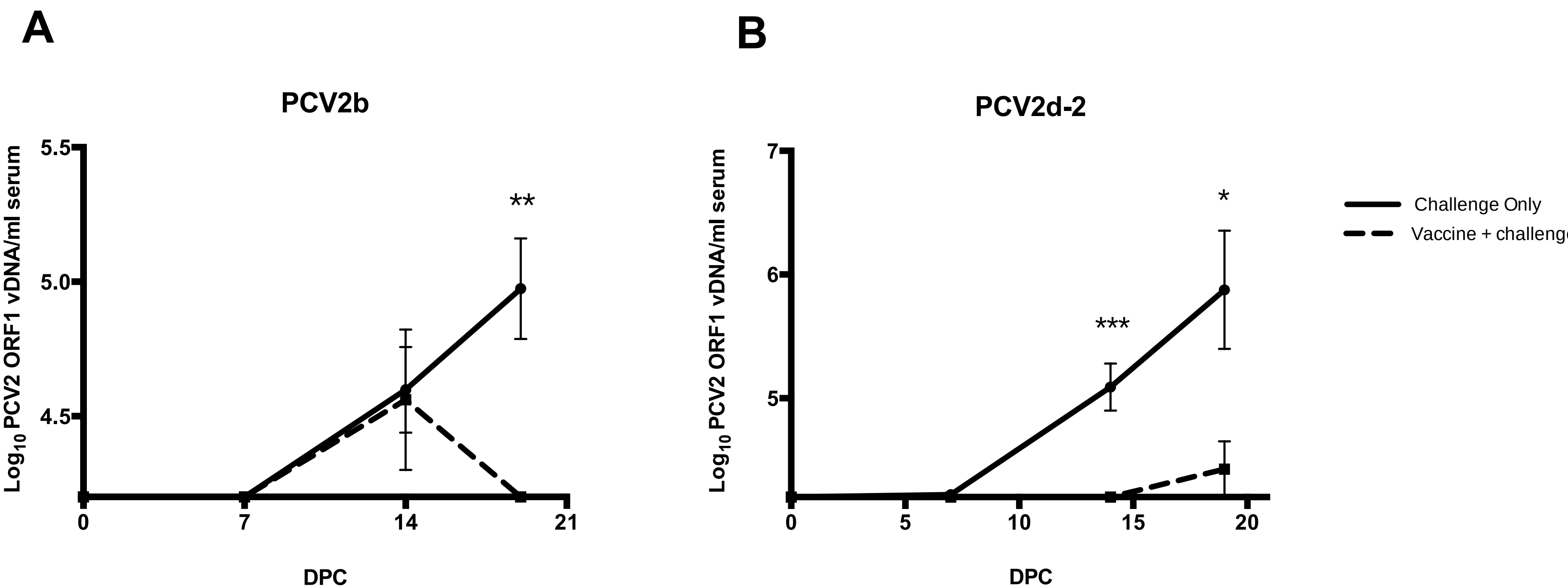

A
PCV2b
5.5
5.0
4.5
**
Log10 PCV2 ORF1 vDNA/ml serum
0
7
14
21
DPC
B
PCV2d-2
7
6
5
*
***
Log10 PCV2 ORF1 vDNA/ml serum
0
5
10
15
20
DPC
Challenge Only
Vaccine + challenge

Figure 6

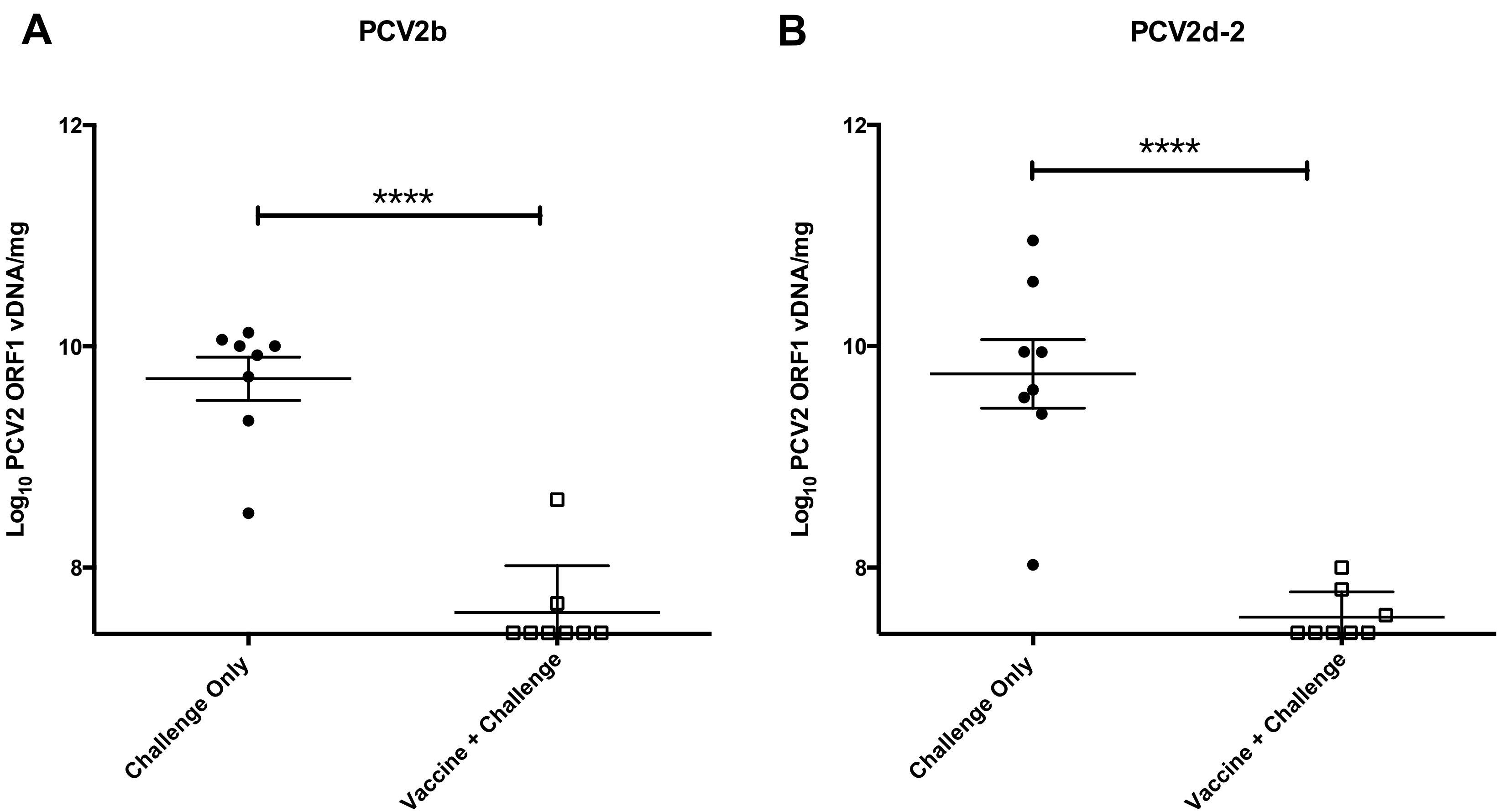

A
PCV2b
B
PCV2d-2
Log10 PCV2 ORF1 vDNA/mg
12
10
8
****
Challenge Only
Vaccine + Challenge

Figure 7

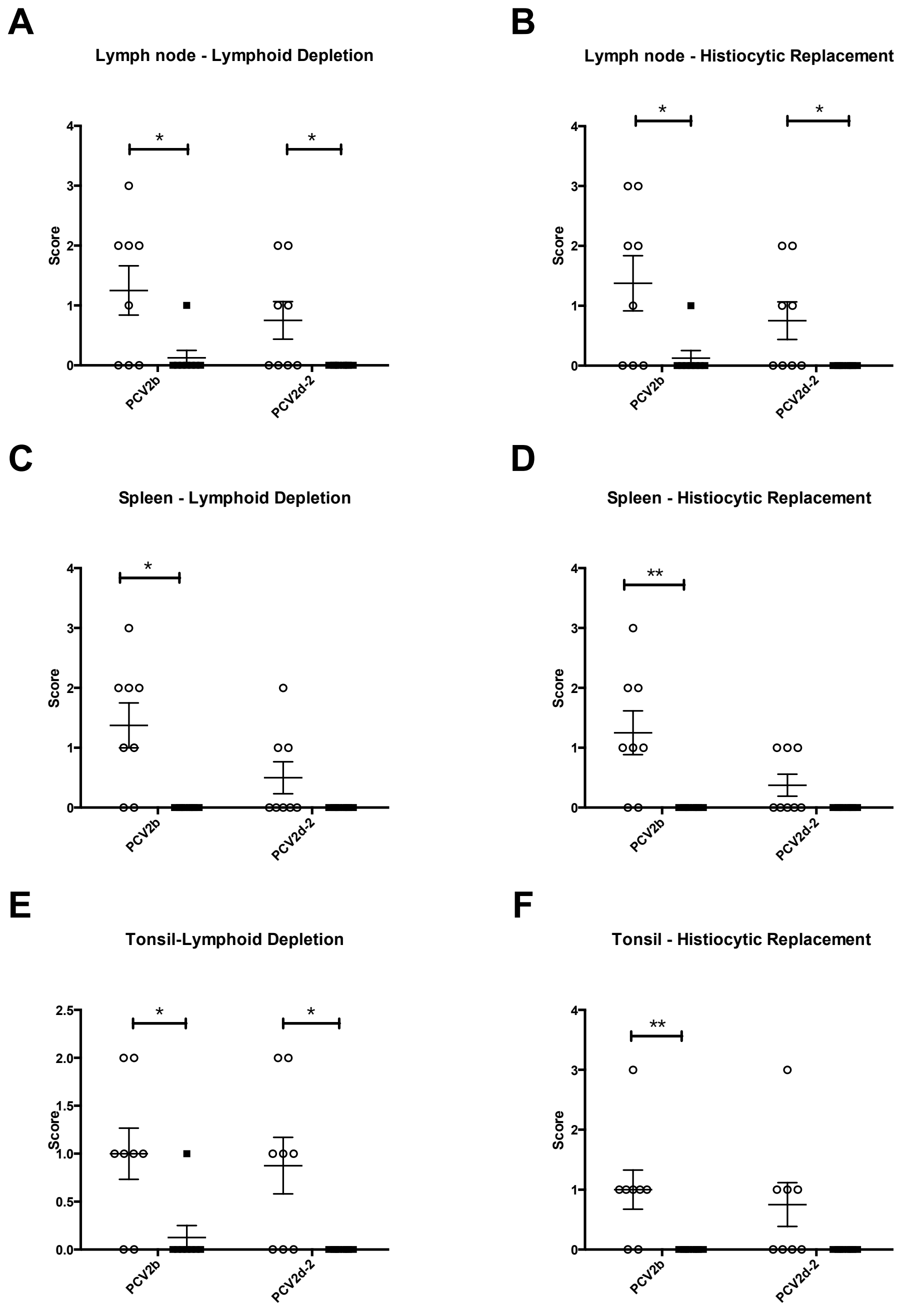

A
Lymph node - Lymphoid Depletion
Score
PCV2b
PCV2d-2
B
Lymph node - Histiocytic Replacement
Score
PCV2b
PCV2d-2
C
Spleen - Lymphoid Depletion
Score
PCV2b
PCV2d-2
D
Spleen - Histiocytic Replacement
Score
PCV2b
PCV2d-2
E
Tonsil-Lymphoid Depletion
Score
PCV2b
PCV2d-2
F
Tonsil - Histiocytic Replacement
Score
PCV2b
PCV2d-2

Figure 8

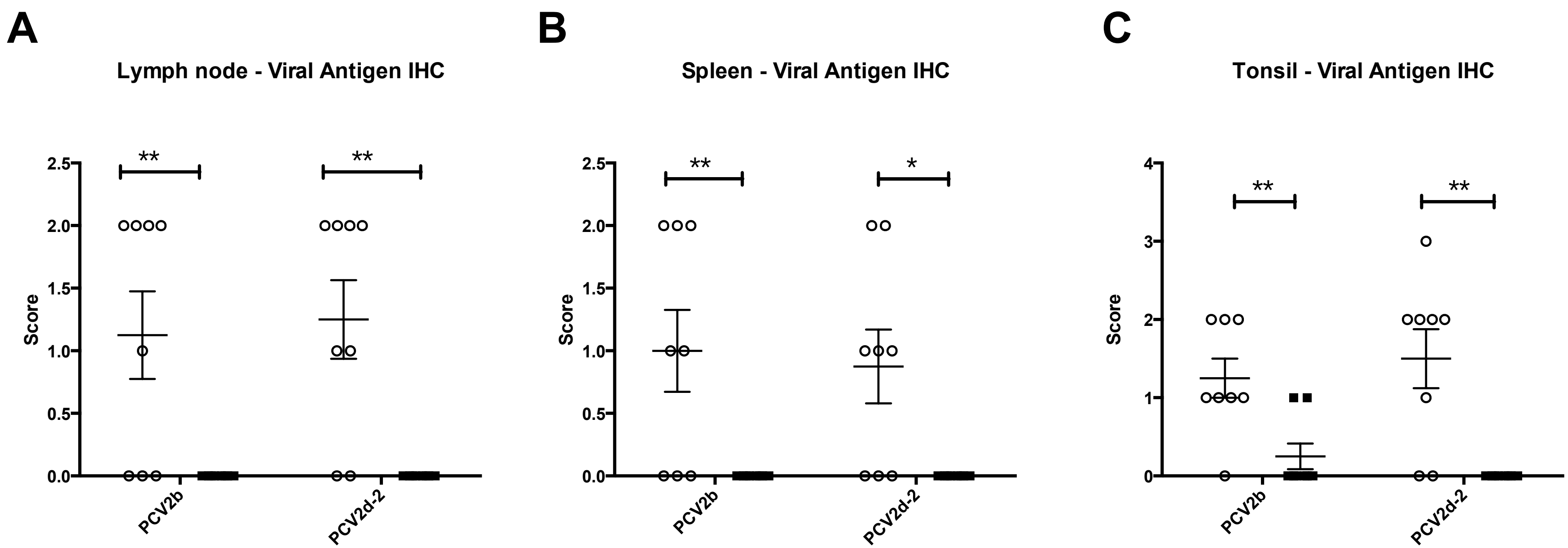

A
Lymph node - Viral Antigen IHC
Score
2.5
2.0
1.5
1.0
0.5
0.0
**
**
PCV2b
PCV2d-2
B
Spleen - Viral Antigen IHC
Score
2.5
2.0
1.5
1.0
0.5
0.0
**
*
PCV2b
PCV2d-2
C
Tonsil - Viral Antigen IHC
Score
4
3
2
1
0
**
**
PCV2b
PCV2d-2

Table 1

Table 1. Seroconversion to PCV2-specific antibodies in pigs experimentally infected with chimeric PCV2 viruses containing shuffled capsids or with the PCV1-2a vaccine virus

Group	Inocula	No. of pigs positive for PCV2 antibodies/total on DPV[a]:							
		0	7	14	21	28	35	42	49
1	PCV2-3cl13	0/3	0/3	0/3	0/3	0/3	1/3	2/3	1/3
2	PCV2-3cl14	0/3	0/3	0/3	0/3	1/3	3/3	3/3	3/3
3	PCV2-3cl4_2	0/3	0/3	0/3	0/3	0/3	1/3	1/3	1/3
4	PCV2-3cl12_2	0/3	0/3	0/3	0/3	0/3	1/3	1/3	2/3
5	PCV1-2a	0/3	0/3	0/3	0/3	1/3	2/3	3/3	3/3
6	PBS	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3

[a] PCV2 antibody was measured at different days post-inoculation (DPI) with an ELISA using the recombinant PCV2 capsid protein as the antigen. Animals were considered to have seroconverted when samples from two or more consecutive time points were seropositive. Seropositive time points are shown in grey.

Table 2

Table 2. Seroconversion to PCV2-specific antibodies by ELISA and detection of viremia by PCR in pigs vaccinated with PCV1-3cl14 virus and challenged with PCV2b or PCV2d-2

Group	Vaccine	Challenge Virus	No. of pigs positive for PCV2 antibodies/total on DPV[a]:							No. of pigs positive for PCV2 antibodies/total on DPC[a,c]:			No. of pigs positive for viremia/total on DPC[b,c]:		
			0	7	14	21	28	35	42	7	14	20	7	14	20
1	PCV1-3cl.14	PCV2b	0/8	0/8	0/8	0/8	1/8	3/8	3/8	7/8	8/8	8/8	0/8	2/8	0/8
2	PCV1-3cl.14	PCV2d-2	0/8	0/8	0/8	0/8	0/8	2/8	4/8	5/8	5/8	5/8	0/8	1/8	2/8
3	None (PBS)	PCV2b	0/8	0/8	0/8	0/8	0/8	0/8	0/8	2/8	5/8	8/8	0/8	4/8	7/8
4	None (PBS)	PCV2d-2	0/8	0/8	0/8	0/8	0/8	0/8	0/8	1/8	4/8	6/8	1/8	7/8	7/8

[a] PCV2 antibody was measured with an ELISA with the recombinant PCV2 capsid antigen. Animals were considered to have seroconverted when samples from two or more consecutive time points were seropositive

[b] Results represent detection by real-time PCR of wild-type PCV2 DNA

[c] At 42 days post-vaccination (DPV), the animals in all four groups were challenged with the wild-type PCV2 virus indicated above

Supplementary Table 1

Table S1: ***Oligonucleotide primers used in this study***

Primer ID	*Primer Sequence (5'-->3')*
uniRep_F	TTACTGAGTCTTTTTTATCACTTCGTAATGG
2aORF2_R	CTTTCGTTTTCAGATATGACGTATCCAAGGAGGCG
uniRep_R	ACCCATTACGAAGTGATAAAAAAGACTCAG
SacII_uni_R	AGCCCGCGGAAATTTCTGACAAACGTTAC
SacII_uni_F	TTTCCGCGGGCTGGCTGAACTTTTGAAAG
PCV1_DSORF2_F	CTTTTTTGTTATCACATCGTAATGGTTTTTATT
PCV1_DSORF2_R	TTCTTTCACTTTTATAGGATGACGTATCCAAGGA
PCV1_BB_F	CCTCCTTGGATACGTCATCCTATAAAACTGAAAGAA
PCV1_BB_R	AAATAAAAACCATTACGATGTGATAACAAAAAAG
NB-56-m2b	GAGGTGTTCGGCCCTCCTCA
PCV2-83F	AAAAGCAAATGGGCTGCTAA
PCV2-83R	TGGTAACCATCCCACCACTT
PCV1 qRepF	TGGAGAAGAAGTTGTTGT
PCV1 qRepR	TCTACAGTCAATGGATACC